Effects of growth and aging on the reference values of pulmonary nitric oxide dynamics in healthy subjects

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Abstract: The lung just like all other organs is affected by age. The lung matures by the age of 20 and age-related changes start around middle age, at 40-50 years. Exhaled nitric oxide (FENO) has been shown to be age, height and gender dependent. We hypothesize that the nitric oxide (NO) parameters alveolar NO (CANO), airway flux (JawNO), airway diffusing capacity (DawNO) and airway wall content (CawNO) will also demonstrate this dependence. Data from healthy subjects were gathered by the current authors from their earlier publications in which healthy individuals were included as control subjects. Healthy subjects (n = 433) ranged in age from 7 to 78 years. Age-stratified reference values of the NO parameters were significantly different. Gender differences were only observed in the 20-49 age group. The results from the multiple regression models in subjects older than 20 years revealed that age, height and gender interaction together explained 6% of variation in FENO at 50 ml s-1 (FENO50), 4% in JawNO, 16% in CawNO, 8% in DawNO and 12% in CANO. In conclusion, in this study we have generated reference values for NO parameters from an extended NO analysis of healthy subjects. This is important in order to be able to use these parameters in clinical practice.

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Abstract

The lung just like all other organs is affected by age. The lung matures by the age of 20 and age-related changes start around middle age, at 40–50 years. Exhaled nitric oxide (F_ENO) has been shown to be age, height and gender dependent. We hypothesize that the nitric oxide (NO) parameters alveolar NO (C_ANO), airway flux ($J_{aw}NO$), airway diffusing capacity ($D_{aw}NO$) and airway wall content ($C_{aw}NO$) will also demonstrate this dependence. Data from healthy subjects were gathered by the current authors from their earlier publications in which healthy individuals were included as control subjects. Healthy subjects (n = 433) ranged in age from 7 to 78 years. Age-stratified reference values of the NO parameters were significantly different. Gender differences were only observed in the 20–49 age group. The results from the multiple regression models in subjects older than 20 years revealed that age, height and gender interaction together explained 6% of variation in F_ENO at 50 ml s^{-1} (F_ENO_{50}), 4% in $J_{aw}NO$, 16% in $C_{aw}NO$, 8% in $D_{aw}NO$ and 12% in C_ANO. In conclusion, in this study we have generated reference values for NO parameters from an extended NO analysis of healthy subjects. This is important in order to be able to use these parameters in clinical practice.

Introduction

The use of non-invasive methods to diagnose respiratory diseases and monitor treatment is advantageous for both patients and healthcare professionals. Exhaled nitric oxide (F_ENO) has been used extensively since its discovery in human breath [1], especially in asthma where clinical practice guidelines have already been published [2]. The pulmonary nitric oxide dynamics models have the advantage of being a more precise assessment of nitric oxide (NO) dynamics, but their application has been limited [3]. The technical development has rapidly evolved and today we have NO analysers adopted for clinical use, both in specialized respiratory medicine and primary care [4, 5].

F_ENO from one single exhalation will give a measured value of NO production from the entire respiratory system. A more detailed insight can be gained through the use of the mathematical two-compartment model (2CM) of pulmonary NO dynamics, which differentiates the NO exchange of the peripheral and central parts of the lung and explains the flow dependence of F_ENO [6, 7]. In brief, the 2CM consists of an alveolar compartment comprising the peripheral gas exchanging parts of the lung (respiratory bronchioles and alveoli) and an airway compartment comprising the conductive

airways larger than respiratory bronchioles. Gas in the alveolar compartment holds a certain concentration of NO (C_ANO). During exhalation, alveolar gas travels through the bronchial compartment and more NO diffuses from the bronchial wall into the luminal gas (airway NO flux, $J_{aw}NO$) [8]. C_ANO and $J_{aw}NO$ can be estimated based on a linear model if F_ENO is measured at three flow rates at of least 100 ml s^{-1} [9]. If a flow rate less than 30 ml s^{-1} is used together with a median and a high flow rate, i.e. 100 and 300 ml s^{-1}, then a non-linear model can be applied which also estimates the airway wall concentration of NO ($C_{aw}NO$) and the diffusing capacity of NO from the airway wall to the gas stream ($D_{aw}NO$) [8, 10]. Investigations have used the 2CM with interesting results, especially for C_ANO where increased values have been found in severe asthma [11], alveolitis [12], and chronic obstructive pulmonary disease [10, 13] and early scleroderma [14]. C_ANO has been specifically used to identify scleroderma patients at high risk for lung function deterioration and advancing disease, with 5.3 ppb being suggested as the cut off value [15].

Reference values are necessary for any new method to be accepted in clinical practice, and reference values for F_ENO at the recommended flow of 50 ml s^{-1} (F_ENO_{50}) have been published [16, 17]. Height, age and gender have been shown to influence F_ENO_{50}. Reference values for NO parameters from extended NO analysis are limited to two publications, one with 89 adults [18] and one with 66 children [19]. The lung matures by the age of 20 in regard to closing volume [20] and in older age the diffusing capacity declines in a linear fashion with increasing age [21], and these changes in pulmonary physiology might also affect NO parameters. The aim of this study was to establish reference values for NO parameters in healthy subjects ranging from young to old age.

Methods

Subjects

Data from healthy non-smoking subjects were gathered by the current authors from their earlier publications in which healthy individuals were included as control subjects [10, 14, 18, 19, 22–30]. In the majority of these studies measurements of lung function and symptom questionnaires verified the health status. Gender, age and height were noted. The exhaled flow together with corresponding exhaled NO levels were collected.

NO analysis

F_ENO_{50} and F_ENO values from exhalation with flows of 5–500 ml s^{-1} for the extended NO analysis were gathered. All data were recalculated either with the linear model (Tsoukias & George, TG) [9] using three flow rates of at least 100 ml s^{-1} or with the non-linear model (Högman-Meriläinen Algorithm, HMA) [10, 22] using a low flow rate of 5, 10 or 20, a median rate of 100 and a high flow rate of 300, 400 or 500 ml s^{-1}. Data were fed into an algorithm in a standard Microsoft® Excel environment, available as supplementary information, for the estimation of the NO parameters (stacks.iop.org/JBR/11/047103/mmedia). When generating NO parameters from the linear model [9], Pearson's r-value was noted. With the use of NO parameters from the non-linear model [10, 22] a plot of flow with corresponding NO values can be generated; at a flow of 50 ml s^{-1}, a NO value was noted and compared to the measured F_ENO_{50} for a quality control of the estimation of the NO parameters. With the non-linear model there is also a built-in quality test of the curve [10]. This is in line with the first guidelines for the extended NO analysis [31].

Statistical analysis

Due to aging of the lung, the subjects were divided into three age groups, <20 years, 20–49 years and $\geqslant 50$ years. Descriptive data of the subjects are presented as frequency or as medians and quartiles where appropriate. The distributions of the NO parameters, stratified by age groups, are presented as an arithmetical mean or geometrical mean (for skewed distributed data) and as 2.5th, 5th, 25th, 50th, 75th, 95th, 97.5th percentiles. A Kruskal-Wallis test and one-way ANOVA were used to test for differences in the distribution of NO parameters between the age groups. In the case of significant difference between age groups, post-hoc tests were performed using a pairwise Mann-Whitney U-test. Pearson Correlation was used to test correlations to C_ANO. Spearman's rank order correlation was used for the other NO parameters.

Gender-stratified simple regression models were fitted with the logarithms of F_ENO_{50}, $C_{aw}NO$, $D_{aw}NO$, and $J_{aw}NO$, respectively, as the dependent variable, and with age as an independent variable. Logarithmically scaled regression lines were retransformed back into natural scale and all regression lines were then plotted along with their corresponding 95% reference intervals.

Multiple regression modelling was performed on data where subjects younger than 20 years were excluded, as children differ from adults in regards to the relationship between age and height, which made if difficult to fit robust statistical models. The models were specified with the C_ANO in natural scale, the logarithms of F_ENO_{50}, $C_{aw}NO$, $D_{aw}NO$, and $J_{aw}NO$, respectively, as the dependent variable, and with age, height and gender, including interaction terms between gender*height and gender*age, as independent variables. For all the models, ANOVA chunk tests were performed, jointly testing if the two interaction terms contributed significantly to the models as compared to omitting them from the model. As this was not the case for any of the NO parameters, the models were refitted without the interaction terms. To

Table 1. Subject characteristics in the different age groups presented by gender.

Age group	<20 yrs		20–49 yrs		⩾50 yrs	
Gender	Female	Male	Female	Male	Female	Male
Subjects, n	41	42	82	113	42	113
Age, years	10 (9, 11)	10 (8, 12)	33 (26, 40)	39 (30, 44)	53 (52, 60)	56 (52, 65)
Height, m	1.39 (1.32, 1.47)	1.37 (1.31, 1.49)	1.68 (1.64, 1.71)	1.81 (1.75, 1.85)	1.67 (1.63, 1.69)	1.76 (1.72, 1.80)
Weight, kg	34 (30, 38)	32 (28, 39)	60 (55, 68)	80 (73, 87)	70 (61, 76)	79 (73, 88)
BMI, kg/m^2	17 (16, 19)	17 (16, 19)	22 (20, 23)	25 (23, 26)	25 (23, 27)	26 (24, 28)

Data are given in median (25, 75 percentile).

account for a potential cluster effect in the data, we also controlled for study centre and estimation method (TG versus HMA). To help the interpretability of regression coefficients, the variables age and height were centred and age was scaled to a unit of 10 years and 10 cm respectively [32]. For the factor gender, B represents the expected ratio in geometrical means between a male and a female, keeping all other variables fixed. For the logarithmically transformed parameters, regression coefficients have been retransformed to natural scale using the exponential function. The bootstrap procedure produces optimism-corrected estimates of R^2, with a correction factor based on the average difference, in over 5000 bootstrap samples, between the R^2 of the model fit to the bootstrap data and the R^2 of the bootstrap model applied to the original data.

Model assumptions of normality and homoscedasticity of residuals were assessed from graphs. A p-value <0.05 was considered statistically significant. Excel (Microsoft^R Office 2011) was used for calculations of the NO parameters. Statistical analyses were performed using SPSS, v. 22 (SPSS Inc., Chicago, MI, USA), and R [33] using the rms package [34].

Results

Healthy subjects (n = 433) aged between 7–78 years were analysed. There were more men (n = 268) than women (n = 165) (table 1). There was no difference in F_ENO_{50} between the study centres (p = 0.37).

The NO parameters were estimated using the linear model TG (n = 87) with an r-value from 0.90 to 1.0, and with a median value of 1.0 (0.99, 1.0). In the non-linear model HMA (n = 346), all passed the built-in quality test. The difference in measured and estimated F_ENO_{50} ranged from −5.0 to 5.0, with a median value of 0.3 (−0.6, 1.3) ppb.

NO parameters in the different age groups

There were statistically significant differences in the distribution of the NO parameters between the young, middle and older age groups (table 2). F_ENO_{50} was higher in the older age group compared to the young age group (p < 0.001) and the middle age group (p = 0.001), and F_ENO_{50} was higher in the middle age group than the younger age group (p < 0.001). $J_{aw}NO$ was lower in the young age group compared to the middle age (p < 0.001) as well as the older age group (p < 0.001). $C_{aw}NO$ was higher in the older age group compared to the young age group (p < 0.001) and the middle age group (p < 0.001), and $C_{aw}NO$ was higher in the middle age group than in the younger age group (p < 0.001). $D_{aw}NO$ was lower in the older age group compared to the young age group (p = 0.023) and the middle age group (p = 0.001). C_ANO was lower in the middle age group compared to the young age group (p = 0.001) and the older age group (p < 0.001).

NO parameters in the different age groups by gender

There was only a difference between genders in the middle age group in F_ENO_{50} (p < 0.001), $J_{aw}NO$ (p < 0.001), $C_{aw}NO$ (p < 0.001) and C_ANO (p = 0.027) but not in $D_{aw}NO$ (table 3).

Regression analyses

Relationships between age and the NO parameters ($J_{aw}NO$, C_ANO, $D_{aw}NO$ and $C_{aw}NO$), with univariate regression lines and estimated 95% reference intervals, are shown in figure 1. F_ENO_{50} is shown in the supplementary material, available online.

The multiple regression analyses, with the bootstrap validation step, showed in the age groups above 20 years that age, height and gender interactions together explained 6% of variation in F_ENO_{50}, 4% in $J_{aw}NO$, 16% in $C_{aw}NO$, 8% in $D_{aw}NO$ and 12% in C_ANO (table 4). Age was a significant predictor in all models (p < 0.001) except for $J_{aw}NO$ (p = 0.18) (table 4). The association was positive for F_ENO_{50} and all NO parameters. Gender contributed as a significant main effect for $C_{aw}NO$ and C_ANO only. Multiple linear regression models poorly predicted the large variations in F_ENO_{50} and NO parameters.

In the age group <20 years there were only 83 subjects and therefore multiple regression models were not applied. Age correlated positively to F_ENO_{50} (r = 0.31, p = 0.005) and to $J_{aw}NO$ (r = 0.32, p = 0.003). There were stronger correlations between height and F_ENO_{50} (r = 0.45, p < 0.001), and height and $J_{aw}NO$ (r = 0.41, p = 0.001), while no correlations were found between height and C_ANO, $C_{aw}NO$ and $D_{aw}NO$.

Table 2. Mean values and percentile distribution of F_ENO_{50} and NO parameters in the three age groups.

			Percentile distribution						
Age groups	Mean	p-value*	2.5	5	25	50	75	95	97.5
[1]F_ENO_{50}, ppb									
<20 yrs	10.8	<0.001	4.7	4.9	7.1	10.5	15.9	25.6	27.0
20–49 yrs	16.0		6.6	7.4	12.0	15.3	20.9	38.0	45.5
⩾50 yrs	18.2		7.7	8.5	13.2	18.2	25.3	36.5	44.9
[1]$J_{aw}NO$, nl/s									
<20 yrs	0.40	<0.001	0.08	0.10	0.26	0.38	0.66	1.36	1.60
20–49 yrs	0.76		0.31	0.34	0.53	0.70	1.08	2.01	2.51
⩾50 yrs	0.81		0.27	0.31	0.52	0.83	1.23	2.00	2.23
[1]$C_{aw}NO$, ppb									
<20 yrs	64	<0.001	19	21	34	58	123	208	439
20–49 yrs	105		30	35	65	105	160	309	441
⩾50 yrs	155		40	51	89	150	267	491	535
[1]$D_{aw}NO$, ml/s									
<20 yrs	7.5	0.002	0.9	1.3	4.8	8.7	13.1	25.6	26.9
20–49 yrs	7.8		1.3	2.5	5.3	8.3	12.7	19.1	21.6
⩾50 yrs	5.7		1.0	1.2	3.5	6.2	10.3	17.1	20.8
C_ANO, ppb									
<20 yrs	2.07	<0.001	0.11	0.61	1.52	2.05	2.73	3.59	3.88
20–49 yrs	1.72		0.21	0.29	1.13	1.61	2.23	3.66	3.93
⩾50 yrs	2.2		0.33	0.51	1.48	2.25	2.85	3.77	3.88

[1] Data with skewed distribution are given in geometrical mean, * Kruskal-Wallis test for difference in mean values between age groups.

Table 3. F_ENO_{50} and NO parameters in the different age groups presented by gender.

Age group	<20 yrs		20–49 yrs		⩾50 yrs	
Gender	Female	Male	Female	Male	Female	Male
[1]F_ENO_{50}, ppb	11 (8, 16)	10 (7, 15)	13 (10, 17)	18 (13, 23)*	17 (12, 23)	19 (14, 26)
[1]$J_{aw}NO$, nL/s	0.43 (0.28, 0.66)	0.37 (0.23, 0.66)	0.63 (0.44, 0.83)	0.87 (0.60, 1.25)*	0.75 (0.49, 1.14)	0.84 (0.54, 1.26)
[1]$C_{aw}NO$, ppb	76 (48, 130)	54 (30, 84)	77 (54, 115)	126 (77, 211)*	121 (71, 173)	163 (100, 288)
[1]$D_{aw}NO$, ml/s	6.5 (4.0, 10.9)	8.7 (5.5, 17, 4)	8.8 (6.2, 12.8)	7.3 (4.8, 12.7)	7.1 (4.8, 11.4)	5.4 (3.2, 9.8)
C_ANO, ppb	2.12 (1.78, 2.39)	1.98 (1.25, 2.33)	1.99 (1.22, 2.39)	1.52 (1.07, 2.06)*	2.44 (1.25, 2.92)	2.20 (1.45, 2.83)

Data are given in median (25,75 percentile). [1] Geometrical mean. Mann-Whitney U-test for gender differences, * $p < 0.05$.

Discussion

In this study we have generated reference values for NO parameters from an extended NO analysis of healthy subjects. By pooling the healthy subjects' data from earlier published data the values of NO parameters for a large group of subjects can be presented. We have found that age influences F_ENO and all the NO parameters, while gender affects NO parameters only in the middle age group. Multiple linear regression models poorly predicted the large variations in F_ENO_{50} and NO parameters. In the See *et al* paper (n = 13.275) about 10% of the variation in F_ENO was explained by a variety of variables [35], and this is in line with the current results (n = 433) where about 6% of the variation in F_ENO_{50} was explained by age, height, gender, NO model and study centre.

Lung development

In the <20 age group, F_ENO_{50} and the airway NO parameters $J_{aw}NO$ and $C_{aw}NO$ were lower than in the other age groups. This could possibly reflect an increasing mucosal surface area with increasing height and growing lung volumes. This was also present in the study by Jacinto *et al* where the F_ENO_{50} increase breakpoint appeared around 14 years in girls and 16 years in boys [17]. This is in line with the growth of the body, and more specifically the development of the bronchial tree.

Ageing

In the middle and older age groups pulmonary aging seems to increase C_ANO. This possibly reflects decreased diffusivity of gases in the distal portion of the lung, as C_ANO is determined not only by factors producing NO in the lung periphery but also by how much alveolar NO can diffuse into the pulmonary circulation where it is rapidly scavenged by haemoglobin. In older age, the diffusing capacity declines in a linear fashion with increasing age [21] and in elderly healthy subjects there is a decrease in steady-state transfer capacity for carbon monoxide (CO) [36] and NO [37]. There is also an increase in residual volume

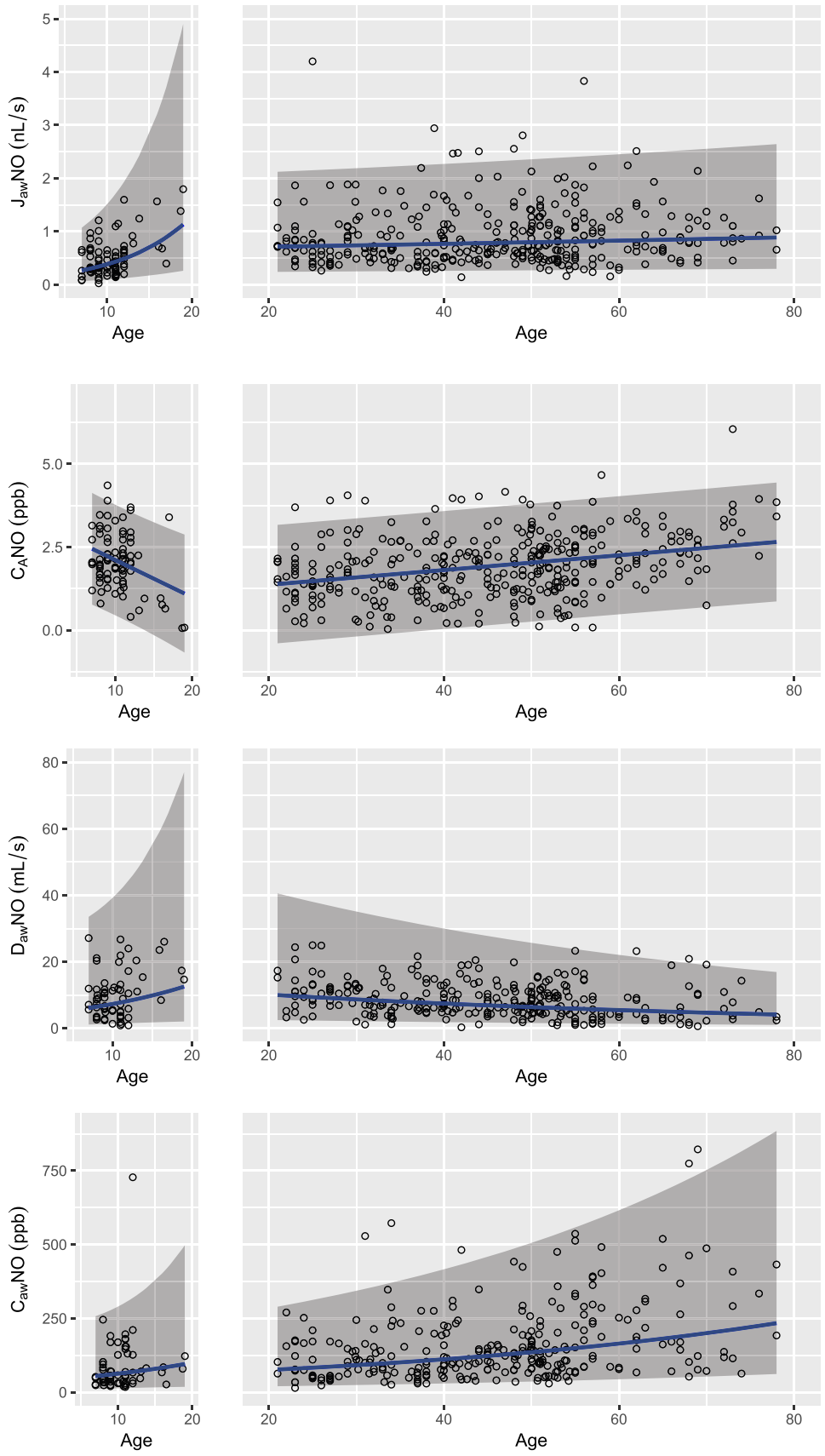

Figure 1. Relationship between age and the NO parameters, airway NO flux ($J_{aw}NO$), alveolar NO (C_ANO), airway diffusing capacity ($D_{aw}NO$) and airway wall content ($C_{aw}NO$), with univariate regression lines and estimated 95% reference intervals. Since children differ markedly from adults, in particular regarding the associations between height and age, the young age group was treated separately.

Table 4. Regression coefficents (B) and p-values of the multiple regression models for NO-variables. The R^2 is the unadjusted coefficient of determination of the models and R^2_{boot} is the corresponding optimism-corrected R^2 values as estimated by bootstrapping.

	Intercept	Age		Height		Gender (male)			
	B	B	p-value	B	p-value	B	p-value	R^2	R^2boot
F_ENO_{50} ppb	15.8	1.07	<0.001	1.04	0.29	1.12	0.13	0.08	0.06
$J_{aw}NO$ nl/s	0.77	1.03	0.18	1.05	0.33	1.10	0.26	0.07	0.04
$C_{aw}NO$ ppb	86.6	1.16	<0.001	0.87	0.04	1.70	<0.001	0.19	0.16
$D_{aw}NO$ ml/s	8.6	0.88	<0.001	1.21	0.01	0.69	0.01	0.11	0.08
C_ANO ppb	1.95	0.2	<0.001	−0.03	0.68	−0.24	0.09	0.15	0.12

[38] reflecting obstruction of the distal part of the airways that could possibly contribute to the increase in C_ANO seen in this study. Thus, there is an accumulation of NO from the alveolar region together with the inhaled NO from the airways that increases with age, and both can contribute to the increase of C_ANO. However, the uptake of NO in pulmonary capillaries is very high [39], and the increase in C_ANO could also be due to other causes. One of these other causes affecting C_ANO may be that clinically healthy older subjects have an altered inflammatory cell profile and can actually have a low-grade inflammation in the lower respiratory tract [40]. This could be due to the macrophages becoming less efficient in scavenging invading microorganisms in older age groups [41, 42]. This could be an explanation for the increased exhaled F_ENO_{50} and NO parameters, i.e. $J_{aw}NO$, $C_{aw}NO$ and C_ANO, in our older subjects.

In studies with older unhealthy patients, it is important that the control subjects be matched to them by age until there is enough data for this age group. Therefore, the increased C_ANO that has been found in COPD patients should be re-evaluated since they have been compared in some studies to younger individuals [10, 13]. However, in other studies, e.g. in cases of systemic sclerosis or alveolitis, the C_ANO values are surely increased since there were no age differences between the patients and control subjects [12, 14, 43]. Matching by gender should also be taken into account for the middle age group, since C_ANO increases earlier in females. This is possibly explained by a decrease in the capillary blood volume of the lung [44] causing an impaired gas exchange in women in the middle age group.

$D_{aw}NO$ decreased with increasing age. This is interesting, as $D_{aw}NO$ is the total diffusivity of NO from bronchial mucosa to luminal air, and it can be assumed to reflect both the total surface area available for diffusion and also the physical properties of the mucosa affecting the diffusivity of gases. As individuals grow so do their bronchial trees, and one would assume that $D_{aw}NO$ increases with increasing height, but we did not see this. Instead, we found that $C_{aw}NO$ increased and this explained the increase in $J_{aw}NO$ and F_ENO_{50} during the growth period. The decrease of $D_{aw}NO$ found in older age might reflect the physical changes occurring in the bronchial mucosa of the aging lung.

Gender

It was only in the middle age group where a gender difference could be found in F_ENO_{50}, $J_{aw}NO$, $C_{aw}NO$ and C_ANO. In the regression model only the variations in $C_{aw}NO$ and C_ANO were significant for gender.

Olin *et al* found F_ENO_{50} to be higher in men than in women around 50 years of age with 18 resp. 15 ppb respectively, but when comparing F_ENO_{50} between the sexes with similar heights and ages no difference was found [16]. Jacinto *et al* have shown a gender difference in the same age group with men slightly above 15 ppb and women around 12 ppb [17]. The corresponding values for F_ENO_{50} in the present study with the young age group excluded are 16 ppb for men and 15 ppb for women, which are in line with the values obtained by Olin *et al* using the same analysing method, namely chemiluminescence.

A limitation in this study is that data were pooled, which resulted in more men than women, especially in the old age group. In addition, the cross-sectional design of the study is not optimal to assess the relation between age and NO parameters. However, long enough longitudinal studies would require decades of follow-up. It would be interesting to put lung function in relation to the NO parameters, but unfortunately we did not have lung function data from all of the subjects. We did check that there was no significant difference in the mean F_ENO_{50} values between the different centres, which suggests that the methodology was similar enough to allow for the pooling of the data.

In conclusion, in this study we have generated reference values for NO parameters from an extended NO analysis of healthy subjects. This is important in order to be able to use these parameters in clinical practice. We found that pulmonary aging seems to increase C_ANO, which is possibly a reflection of a decreased diffusivity of gases in the gas exchange area. The impaired immune defence system that occurs with old age could also explain the increase in all NO parameters except $D_{aw}NO$ that was decreased in this group. Further studies or additional pooling of data are needed before we can provide even better age-related reference values for the NO parameters and

possibly create reliable reference equations. However, this is currently the largest dataset for NO parameters that can be used as a basis for comparisons in future studies regarding health and disease.

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